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Ultracentrifugation of Serum Samples Allows Detection of Hepatitis C Virus RNA in Patients with Occult Hepatitis C▽

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Occult hepatitis C virus (HCV) infection of patients with abnormal liver function tests of unknown origin who are anti-HCV and serum HCV RNA negative but who have HCV RNA in the liver has been described. As HCV replicates in the liver cells of these patients, it could be that the amount of circulating viral particles is under the detection limit of the most sensitive techniques. To prove this hypothesis, serum samples from 106 patients with occult HCV infection were analyzed. Two milliliters of serum was ultracentrifuged over a 10% sucrose cushion for 17 h at 100,000 × g_{av}, where av means average, and HCV RNA detection was performed by strand-specific real-time PCR. Out of the 106 patients, 62 (58.5%) had detectable serum HCV RNA levels after ultracentrifugation, with a median load of 70.5 copies/ml (range, 18 to 192). Iodixanol density gradient studies revealed that HCV RNA was positive at densities of 1.03 to 1.04 and from 1.08 to 1.19 g/ml, which were very similar to those found in the sera of patients with classical chronic HCV infection. Antigenomic HCV RNA was found in the livers of 56 of 62 (90.3%) patients with detectable serum HCV RNA levels after ultracentrifugation, compared to 27 of 44 (61.4%) negative patients ($P < 0.001$). No differences in the median loads of antigenomic HCV RNA between patients with an those without serum HCV RNA (4.5×10^4 [range, 7.9×10^2 to 1.0×10^6] versus 2.3×10^4 [range, 4.0×10^2 to 2.2×10^5]) were found. Alanine aminotransferase and gamma-glutamyl transpeptidase levels, liver necroinflammatory activity, and fibrosis did not differ between both groups. In conclusion, HCV RNA can be detected in the sera of patients with occult HCV infection after circulating viral particles are concentrated by ultracentrifugation.

The viral genome of hepatitis C virus (HCV) is a 9.6-kb linear single-stranded RNA molecule of positive polarity that replicates via an HCV RNA strand of negative polarity (18).

Chronic HCV infection is a progressive disease that may lead to liver cirrhosis and hepatocellular carcinoma (6, 21). The hallmark of chronic hepatitis C is the presence of anti-HCV and HCV RNA in serum for more than 6 months after acute infection. Recently, a new form of chronic HCV infection called "occult HCV infection," characterized by the presence of genomic HCV RNA in liver in the absence of anti-HCV and serum HCV RNA, in patients with abnormal liver function tests of unknown etiology was described (3). In 84% of patients with occult HCV infection, the negative-polarity (antigenomic) HCV RNA strand was also detected in liver, indicating that the virus was replicating. Furthermore, 70% of these patients had HCV RNA in peripheral blood mononuclear cells (PBMCs) (3), and viral replication in these cells was also reported (4).

Since HCV replicates in the livers of patients with occult HCV infection, the reason why HCV RNA is not detected in serum is unknown. One possible explanation is that the number of circulating viral particles is too low to be detected even using the most sensitive reverse transcription (RT)-PCR techniques.

In the present study, by using ultracentrifugation and a sensitive real-time PCR technique, we have demonstrated that sera from patients with occult HCV infection contain low amounts of viral particles and that the physical characteristics of these virions are similar to those found in patients with classical chronic HCV infection.

MATERIALS AND METHODS

Patients. One hundred six patients (74 males) with occult HCV infection were analyzed in this study. Patients had abnormal liver function tests, were anti-HCV negative (INNOTEST HCV Ab IV; Innogenetics, Ghent, Belgium), and did not have serum HCV RNA levels tested by real-time RT-PCR (see below). Other causes of liver diseases (infection by hepatitis B virus, autoimmunity, genetic disorders, alcohol intake, drug toxicity, etc.) were excluded on the basis of clinical, analytical, and epidemiological data. None of the patients were infected with human immunodeficiency virus. All patients had genomic HCV RNA in liver tested by RT-PCR as described previously (3), and its presence was confirmed by in situ hybridization. The epidemiological, biochemical, and histological characteristics of the 106 patients are shown in Table 1. The study was performed in accordance with the principles of the Declaration of Helsinki, and all patients gave their written informed consent to participate in it.

The liver biopsy was obtained from all patients for diagnostic purposes. When the liver fragment was obtained, it was cut into two portions: one was used for histological diagnosis (20), and the second was submerged (no later than 30 s after the liver sample was obtained) into RNAlater (Ambion Inc., Austin, TX) and stored at −20°C until its use for HCV RNA detection. Serum and PBMC samples were collected from all patients on the same day of the liver biopsy. Serum samples were stored at −80°C, while PBMCs were stored in RNAlater (Ambion) at −20°C.

To determine that the lack of anti-HCV antibodies in patients with occult HCV infection was not due to the loss of these antibodies because of cryoglobulin precipitation, anti-HCV was tested again (INNOTEST HCV Ab IV; Innogenetics) in freshly isolated serum samples (not frozen samples) from 40 out of the 106 patients included in the study.

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TABLE 1. Characteristics of the patients[a]

Characteristic	Value
Age (yr)	44.1 ± 11.4
Gender [no. of patients (%)]	
Male	74 (69.8)
Female	32 (30.2)
Known duration of the disease (mo) ± SD[b]	74.3 ± 80.8
Epidemiology [no. of patients (%)]	
Unknown	82 (77.4)
Parenteral	10 (9.4)
Blood transfusion	8 (7.5)
Familial antecedents	6 (5.7)
ALT level (IU/liter) ± SD	85.5 ± 88.9
GGTP level (IU/liter) ± SD	98.7 ± 100.6
Liver inflammation [no. of patients (%)]	
No	56 (52.8)
Yes	50 (47.2)
Steatosis [no. of patients (%)]	
No	75 (70.8)
Yes	31 (29.2)
Fibrosis [no. of patients (%)]	
No	73 (68.8)
Yes	29 (27.4)
Cirrhosis	4 (3.8)

[a] $n = 106$ patients. ALT, alanine aminotransferase; GGTP, gamma-glutamyl transpeptidase.
[b] Time elapsed since abnormal liver function tests were detected for the first time after obtaining the liver biopsy.

Isolation of total RNA from serum, liver, and PBMCs. Total RNA was isolated from 250 μl of serum using TRIzol LS reagent (Invitrogen, Carlsbad, CA). After precipitation, the RNA pellet was dissolved in diethyl-pyrocarbonate (DEPC)-treated water.

Total RNA from PBMCs and liver biopsies was isolated with the SV Total RNA Isolation system (Promega Co., Madison, WI). After precipitation, the pellet was dissolved in DEPC-treated water, and the RNA concentration was determined by spectrophotometry.

HCV RNA detection in ultracentrifuged serum samples. Two milliliters of serum was ultracentrifuged over a 10% sucrose cushion for 17 h at 100,000 × g_{av}, where av means average, and 4°C to concentrate HCV particles. The pellet was dissolved in 250 μl of TE buffer (10 mM Tris-HCl, 10 mM EDTA [pH 7.5]), and total RNA was isolated using TRIzol LS reagent (Invitrogen). After precipitation, the RNA pellet was dissolved in 10 μl of DEPC-treated water, and 5 μl was used for HCV RNA detection by real-time RT-PCR.

Serum HCV-RNA detection by transcription-mediated amplification (TMA). The presence of HCV RNA was tested again using sera from 20 patients with occult HCV infection (10 with and 10 without detectable levels of HCV RNA in serum after ultracentrifugation) by use of a commercial assay based on transcription-mediated amplification (VERSANT HCV RNA Qualitative assay; Bayer Diagnostics, Berkeley, CA) according to the instructions of the supplier.

Phylogenetic analysis. To discard cross-contamination among positive samples, the HCV core region was amplified as described previously (3) by using sera of 10 patients who had HCV RNA-positive results after ultracentrifugation and from whom enough sera were available to repeat the ultracentrifugation step. The 302-bp core PCR products were cloned using the QIAGEN (Hilden, Germany) PCR cloning kit, and four clones from each patient were automatically sequenced. Sequences were aligned with core sequences corresponding to all HCV genotypes retrieved from GenBank by using ClustalX version 1.81 (22). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (12). Genetic distances were estimated using the Kimura two-parameter method, and standard errors of distances were computed by a bootstrap method (1,000 replicates). A phylogenetic tree was constructed with the neighbor-joining method, and its statistical significance was tested by the bootstrap method (1,000 replicates).

Determination of the density of HCV RNA-containing particles. Two milliliters of serum from five patients with occult HCV infection who were positive for HCV RNA in serum after ultracentrifugation and 2 ml of serum from five patients with chronic hepatitis C (anti-HCV and serum HCV RNA positive) were ultracentrifuged as described above. Pellets were resuspended in 250 μl of TE buffer and layered onto discontinuous iodixanol (OptiPrep; Axis-Shield, Oslo, Norway) density gradients (6%, 10%, 20%, 30%, 40%, 50%, and 56.4%; 0.5 ml each) prepared with a solution containing 0.5 M Tris-HCl (pH 8.0), 0.1 M EDTA (pH 8.0), and 0.25 M sucrose, as described previously (14). Gradients were ultracentrifuged at 90,000 × g_{av} and 4°C for 24 h. After ultracentrifugation, 250-μl fractions (15 fractions) were collected from the tops of the gradients. Finally, total RNA was isolated from each fraction using TRIzol LS reagent. After isopropanol precipitation, RNA pellets were resuspended in 10 μl of DEPC-treated water, and the 10 μl was used for HCV RNA detection by real-time RT-PCR.

Quantitative strand-specific real-time RT-PCR. Quantification of HCV RNA negative and positive strands was done by strand-specific real-time RT-PCR using the *Tth* enzyme for the synthesis of cDNA at a high temperature. For detecting genomic HCV RNA, the cDNA was generated with total RNA isolated from serum, 0.5 μg of total RNA isolated from liver, or PBMCs using antisense primer UTRLC2 (5′-CAAGCACCCTATCAGGCAGT-3′) as described previously (5). For antigenomic HCV RNA detection, cDNA was synthesized with sense primer UTRLC1 (5′-CTTCACGCAGAAAGCGTCTA-3′) using 0.5 μg of total RNA isolated from liver (5).

Real-time PCR using fluorescence resonance energy transfer (FRET) probes was run using a Light Cycler (Roche Molecular Biochemicals, Indianapolis, IN) with 2 μl of cDNA in a final volume of 20 μl containing 5 mM $MgCl_2$, 0.5 μM of primers UTRLC1 and UTRLC2, 0.5 μM of each probe, and 2 μl LightCycler FastStart DNA Master HybProbe mix (Roche Molecular Biochemicals). Probes described previously by Bullock et al. (2) were used: FRET anchor probe HCVG-fluorescein isothiocyanate (FITC) (5′-GCCATAGTGGTCGTCTGCG GAACCGGT-FITC-3′) and FRET sensor probe RED-HCVG (5′-LCRed640-GTACACCGGAATTGCCAGGA-phosphate-3′).

Amplification was performed as follows: initial denaturation and activation of the enzyme for 10 min at 95°C, followed by 60 cycles of denaturation at 95°C for 1 s, annealing at 55°C for 12 s with a single measurement of fluorescence at the end of the annealing step, and elongation at 72°C for 12 s. After amplification, melting curve analysis was performed by heating the samples at 95°C for 5 s, cooling to 40°C for 30 s, and heating to 80°C with a temperature transition rate of 0.1°C/s and continuous fluorescence data acquisition.

Two standard curves constructed with synthetic HCV RNA of positive or negative polarity were performed for the quantification of the genomic or antigenomic HCV RNA. The linearity of the quantification assay ranged from 3.2 to 3.2×10^8 copies of HCV RNA positive or negative strand per reaction. This assay was capable of detecting 3.2 molecules of the correct strand while unspecifically detecting 10^7 to 10^8 copies of the incorrect strand. Sensitivities and dynamics of each assay were not affected when total RNA extracted from HepG2 cells was added to the reaction mixture.

In situ hybridization. Genomic HCV RNA was detected as described previously (7, 9, 16), with a cRNA probe labeled with digoxigenin 11-UTP obtained by in vitro transcription of pC5′NCR, which contains the complete 5′ noncoding region of the HCV genome. The percentage of infected cells was determined by visual inspection, and at least 2,000 cells from each liver section were counted.

Statistical analysis. Statistical analysis was done using SPSS version 9.0 (SPSS Inc., Chicago, IL). Continuous variables were expressed as means ± standard deviations or as medians and ranges, and comparisons were performed with the Student *t* test or the Mann-Whitney U test. Categorical variables were expressed as a percentage and were compared with the χ^2 test. A two-sided *P* value of ≤0.05 was considered to be statistically significant.

Nucleotide sequence accession numbers. Sequences of the HCV core PCR products are available from GenBank under accession numbers DQ839181 to DQ839220.

RESULTS

Anti-HCV antibodies in unfrozen serum samples. Anti-HCV antibodies were not detected in any of the freshly isolated serum samples (samples that were never frozen) from the 40 patients with occult HCV infection.

HCV RNA detection in total RNA directly isolated from serum samples. No HCV RNA was detected in any of the 106 serum samples by real-time RT-PCR when the viral RNA was tested in total RNA directly isolated from 250 μl of serum.

Detection of HCV RNA in ultracentrifuged serum samples. Among the 106 patients included in the study, HCV RNA was detected in 62 (58.5%) of them after ultracentrifugation of

TABLE 2. Comparison of characteristics between patients with and those without HCV RNA in serum after ultracentrifugation

Characteristic[a]	Value for group		*P* value
	Negative[b]	Positive[c]	
Age (yr)	46.0 ± 12.4	42.8 ± 10.6	0.150
Gender [no. of patients (%)]			
Male	33 (75.0)	41 (66.1)	0.327
Female	11 (25.0)	21 (33.9)	
Known duration of disease (mo) ± SD[d]	77.2 ± 68.8	72.2 ± 88.9	0.293
Epidemiology			
Unknown	35 (79.5)	47 (75.8)	0.312
Parenteral	3 (6.8)	7 (11.3)	
Blood transfusion	1 (2.3)	5 (8.14)	
Familial background	5 (11.4)	3 (4.8)	
ALT level (IU/liter) ± SD	86.4 ± 68.3	84.9 ± 101.6	0.168
GGTP level (IU/liter) ± SD	119.3 ± 122.8	84.1 ± 79.1	0.238

[a] ALT, alanine aminotransferase; GGTP, gamma-glutamyl transpeptidase.
[b] $n = 44$.
[c] $n = 62$.
[d] Time elapsed since abnormal liver function tests were detected for the first time after obtaining the liver biopsy.

TABLE 3. Comparison of histologic characteristics between patients with and those without HCV RNA in serum after ultracentrifugation

Characteristic	Value for group		*P* value
	Negative[a]	Positive[b]	
Liver inflammation			
No	26 (59.1)	30 (48.4)	0.277
Yes	18 (40.9)	32 (51.6)	
Steatosis			
No	27 (61.4)	48 (77.4)	0.078
Yes	17 (38.6)	14 (22.6)	
Fibrosis			
No	33 (75.0)	40 (64.5)	0.398
Yes	9 (20.5)	20 (32.3)	
Cirrhosis	2 (4.5)	2 (3.2)	

[a] $n = 44$.
[b] $n = 62$.

serum samples, with a median viral load of 70.5 copies/ml (range, 18 to 192). No differences between patients with and those without detectable levels of HCV RNA in serum after ultracentrifugation were found when age, gender, known duration of the disease, and alanine aminotransferase and gamma-glutamyl transpeptidase levels were compared (Table 2). With respect to histological damage, no statistical differences in liver inflammation, steatosis, or fibrosis were found between patients with and those without detectable levels of HCV RNA in serum (Table 3).

In 10 of the patients with HCV RNA that was detectable in serum after ultracentrifugation (viral load ranging from 60 to 160 copies/ml) and in 10 patients for whom viral RNA was undetectable after ultracentrifugation, HCV RNA in serum was tested again by TMA, with negative results in all cases.

HCV core sequencing and phylogenetic analysis. Sequencing of HCV core PCR products obtained from the 10 patients for whom enough serum was available to repeat ultracentrifugation and phylogenetic analyses showed that the clones segregated separately in the 10 cases, indicating that no cross-contamination occurred and that all sequenced HCV isolates were grouped together with the sequences of genotype 1b (Fig. 1).

HCV RNA in ultracentrifuged serum samples and PBMCs. HCV RNA was detected after ultracentrifugation of serum samples from 24 patients who were HCV RNA positive in PBMCs. On the other hand, viral RNA was found in the PBMCs of 31 patients who did not have HCV RNA in their ultracentrifuged serum samples. Finally, 38 patients were simultaneously HCV RNA positive in the ultracentrifuged serum and in PBMCs. Overall, detection of HCV RNA in serum after ultracentrifugation and in PBMCs made it possible to diagnose occult HCV infection in 93 (87%) out of the 106 patients included in the study.

Distribution of HCV RNA in iodixanol density gradients. Real-time PCR analysis of the fractions obtained from the iodixanol density gradients showed that in the sera from the five patients with occult HCV infection, HCV RNA appeared in two different peaks: the first one at densities of 1.03 to 1.04 g/ml and the second peak at densities ranging from 1.08 to 1.19 g/ml. These densities were similar to those at which the viral RNA was found in sera from the five patients with classical chronic HCV infection (1.03 g/ml to 1.04 g/ml and from 1.07 to 1.30 g/ml). An example of the distribution of HCV RNA in iodixanol density gradients is shown in Fig. 2.

Genomic and antigenomic HCV RNA in liver biopsies. All the patients were HCV RNA positive by liver biopsy, with a median concentration of genomic HCV-RNA strand of 2.6×10^5 copies/μg total RNA (range, 3.3×10^3 to 3.6×10^6). Melting curve analysis of PCR products showed that the HCV infecting the liver of these patients belonged to HCV genotype 1. No differences in the concentrations of genomic HCV RNA strand were found between the patients with and those without HCV RNA in serum after ultracentrifugation (2.1×10^5 copies/μg total RNA [range, 2.2×10^4 to 1.3×10^6] versus 3.2×10^4 copies/μg total RNA [range, 3.3×10^4 to 1.5×10^6]; $P = 0.152$). Antigenomic HCV RNA strands were found in 83 of the 106 (78.3%) patients included in the study, with a median concentration of 3.8×10^4 copies/μg total RNA (range, 2.3×10^4 to 1.0×10^6). Antigenomic HCV RNA strands were found in the livers of 56 (90.3%) out of the 62 patients with detectable HCV RNA levels in serum after ultracentrifugation, while in the patients without detectable viral RNA in serum, the antigenomic strand was positive in the liver biopsies of 27 (61.4%) of the 44 patients, with the differences being statistically significant ($P < 0.001$). No statistical differences in the median concentrations of the antigenomic HCV RNA strand were found between both groups of patients (HCV RNA positive in ultracentrifuged serum, 4.6×10^4 copies/μg RNA [range, 7.9×10^2 to 1.0×10^6]; HCV RNA negative in ultracentrifuged serum, 2.3×10^4 copies/μg RNA [range: 4.0×10^2 to 2.2×10^5]; $P = 0.253$).

Hepatic HCV RNA by in situ hybridization. In all patients, the presence of HCV RNA in the liver was confirmed by in situ

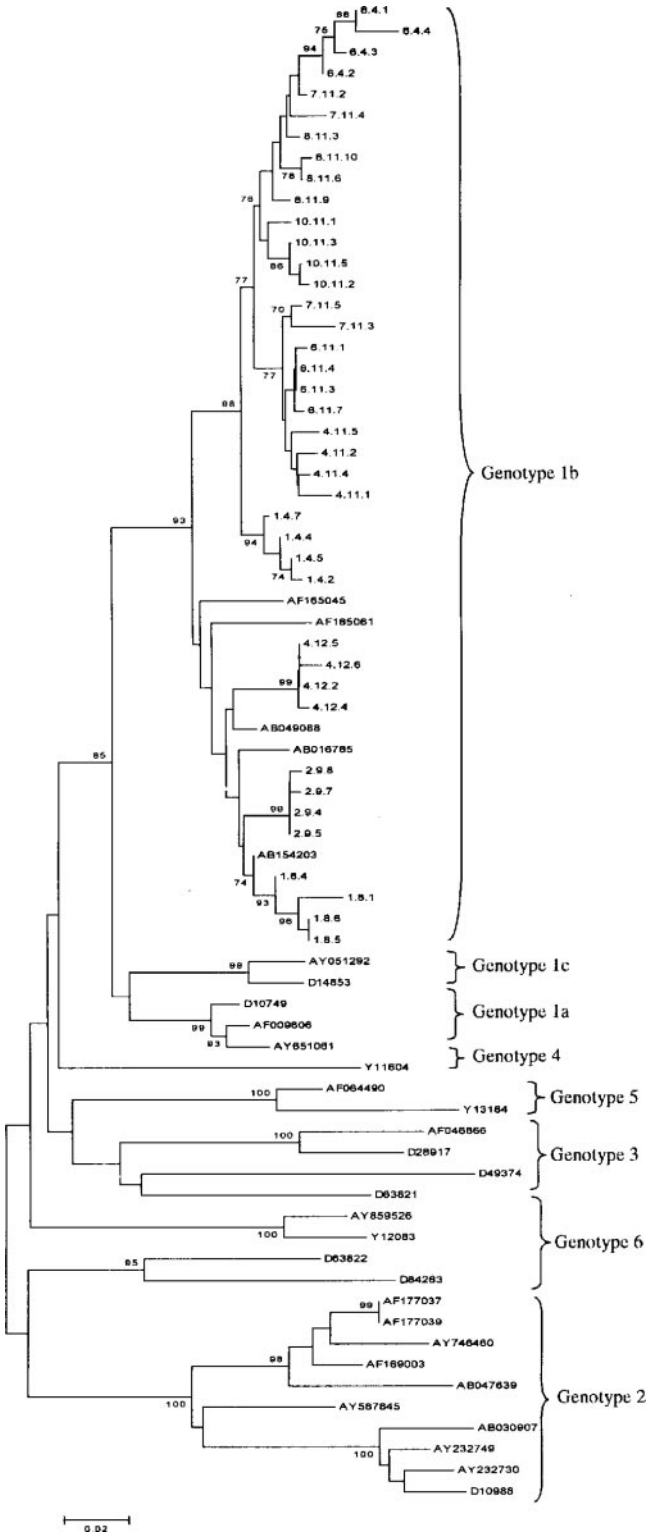

FIG. 1. Neighbor-joining tree constructed with the HCV core nucleotide sequences of the clones of 10 serum HCV RNA-positive patients (GenBank accession numbers DQ839181 to DQ839220) from whom enough serum sample was available to repeat ultracentrifugation and those corresponding to the different HCV genotypes. Bootstrap values ≥70, obtained after 1,000 replicates of the data sheet, are shown in the nodes of the tree.

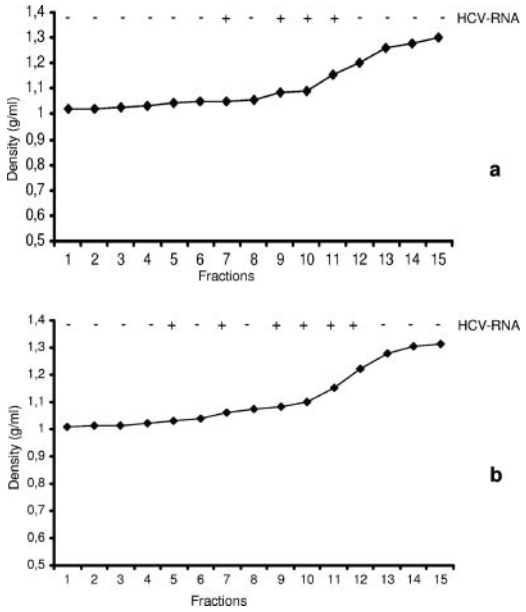

FIG. 2. HCV RNA detection in fractions obtained from the iodixanol gradients in the serum of a patient with an occult HCV infection (a) or with a classical chronic HCV infection (b).

hybridization. The mean percentage of infected hepatocytes in the 106 patients was 4.3% ± 3.9%, and no differences were found between patients with detectable levels of HCV RNA in serum after ultracentrifugation and those who were HCV RNA negative (4.9% ± 4.1% versus 3.9% ± 3.0%; $P = 0.104$).

DISCUSSION

Recently, a new form of HCV infection, called occult HCV infection, was described (3). In these patients, who had abnormal liver function tests of unknown etiology and who are anti-HCV negative, both genomic and antigenomic HCV RNA strands are detected in liver and PBMCs, although HCV RNA is undetectable in serum (3, 4). The reason for the lack of detection of the viral RNA in sera of these patients in whom the virus is replicating in liver and PBMCs is unknown, although it may be speculated that the serum contains low HCV RNA levels that are below the detection limits of sensitive RT-PCR techniques. To verify this hypothesis, we have used ultracentrifugation in order to increase the sensitivity of HCV RNA detection in serum.

When viral RNA is isolated directly from serum, only small volumes of the sample can be easily managed. In fact, in most of the currently used methods for HCV RNA detection, the total RNA is isolated from a maximum of 250 μl of serum (8, 10, 11, 13). By contrast, the use of ultracentrifugation permits the concentration of viral particles from large volumes of serum that would otherwise be difficult to manage for the direct isolation of total RNA, increasing the sensitivity of HCV RNA detection. Thus, the ultracentrifugation of 2 ml of serum would increase the sensitivity of the viral RNA detection at least eight times with respect to techniques in which HCV RNA is directly isolated from serum. In this way, low levels of HCV RNA were detected in the serum of 62 of the 106 (58.5%) patients with

occult HCV infection analyzed in this study. The median viral load was 70.5 copies of HCV RNA per ml of serum (range, 18 to 192 copies/ml). Cross-contamination between samples was ruled out by the phylogenetic analysis performed with the HCV core sequences amplified from 10 patients with detectable levels of HCV RNA in serum.

The utility of the ultracentrifugation of serum samples for HCV RNA detection in patients with occult HCV infection is reinforced by the fact that serum samples from 10 patients who had HCV RNA after ultracentrifugation were negative for viral RNA when tested by TMA, which is one of the most sensitive commercially available tests for HCV RNA detection (19).

Until now, the "gold standard" tool for the diagnosis of occult HCV infection was the detection of HCV RNA in the liver since in PBMCs, viral RNA is detectable in only 70% of patients with this occult infection (3). However, with simultaneous testing for HCV RNA in PBMCs and in ultracentrifuged serum samples, an occult HCV infection can be identified in up to 87% of the cases. Therefore, in light of these results, this is a substantial improvement in the accurate diagnosis of occult HCV infection without the need for a liver biopsy.

Of the 106 patients analyzed, 78.3% had antigenomic HCV RNA in liver. Among the patients with sera that were positive for HCV RNA after ultracentrifugation, antigenomic HCV RNA was found in the livers of 90.3% of the cases, while this occurred in 61.4% of the cases in which viral RNA was undetectable in serum ($P < 0.001$). This finding indicates that the presence of HCV RNA in sera of patients with occult HCV infection is related to the presence of viral replication in liver. Furthermore, median concentrations of both genomic and antigenomic HCV RNA strands as well as the mean percentage of HCV-infected hepatocytes in the liver were higher (although without statistical significance) in patients with HCV RNA in serum than in those in whom the viral RNA was undetectable.

Regarding the density of the serum viral particles containing the viral RNA, ultracentrifugation in iodixanol gradients showed that HCV RNA was detected at densities of 1.03 to 1.04 g/ml and from 1.08 to 1.19 g/ml in serum samples from patients with occult HCV infection. These densities are very similar to those at which HCV RNA was detected in the serum samples from patients with classical chronic HCV infection (1.03 to 1.04 g/ml and from 1.07 to 1.30 g/ml). These results are in agreement with those reported previously by Nielsen et al. (14), who, by analyzing serum samples from patients with chronic HCV infection using iodixanol density gradients, detected HCV RNA at densities below 1.3 g/ml, with most of the viral RNA at densities of $\leq$1.08 g/ml. By using immunoprecipitation, those authors demonstrated that low-density HCV is associated with ApoB and ApoE in particles resembling very-low-density lipoproteins (14). In relation with this, transmission studies of chimpanzees have shown that serum rich in low-density HCV particles associated with host lipoproteins is more infectious than serum rich in high-density particles (1). Thus, all these data considered as a whole suggest that serum from patients with occult HCV infection may contain infectious HCV particles. This finding may have epidemiological implications. Since the acquisition mode of infection is unknown in a proportion of patients with chronic hepatitis C, patients with occult HCV infection may be considered to be a possible source of HCV infection among family members, surgical procedures, etc. However, an epidemiological study on occult HCV infection should be performed to confirm this hypothesis. Regarding clinical implications, since 12% of patients with occult HCV infection may have a fibrosis score of 2 or higher, including liver cirrhosis (3), and may benefit from antiviral therapy (15), detection of HCV RNA in PBMCs and in serum by ultracentrifugation and real-time PCR may be useful in controlling the efficacy of treatment in the majority of cases.

Finally, no differences in either liver enzyme levels or liver damage were found between patients with and those without detectable levels of HCV RNA in serum, indicating that, as occurs in patients with classical chronic HCV infection (16, 17), liver damage in occult HCV infection does not depend on HCV RNA serum levels.

In summary, in the present study, we have demonstrated that a significant proportion of patients with occult HCV infection have low levels of HCV RNA in serum that are detectable only after ultracentrifugation.

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